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Effects of IAA and Myo-inositol on Mitotic Cycles

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Summary

The effects of myo-inositol on cell division and of IAA on the duration of mitotic cycles in root apices meristems have been studied. In V. faba and A. cepa, myo-inositol induces chromosome contraction and lateral adhesion of sister-chromatids and the centromeres are no longer visible as clear unstained gaps; it does not suppress anaphase.

IAA was found to increase the duration of the mitotic cycle of colchicine induced tetraploid cells. 10^{-4} and 10^{-6} M IAA increase intermitotic time by 3-7 hours. Roots were also labeled with H^3 -thymidine (H^3 -TdR) and then treated with 10^{-5} M IAA. Cells in G_2 were not affected but cells in S at the time of treatment with IAA were delayed. From the curves of labeled prophases we have calculated that 10^{-5} M IAA reduces the rate at which cells pass through S for a period of about 7 hours. These results provide further evidence that IAA may be implicated with DNA synthesis and thus, in the control of the duration of mitotic cycles within apical meristems. If IAA prolongs S or can delay the entry of G_1 cells into S, it would provide a mechanism, in roots, for maintaining groups of cells in a quiescent condition.

Introduction

IAA and myo-inositol are two naturally-occurring compounds that are important for plant growth. For both compounds a requirement

for cell proliferation has been demonstrated in vitro. The proliferating systems in which these requirements have been demonstrated include single cell suspensions, calluses, roots developing in culture and tumor cells (Skoog, 1954; Das et al., 1956; Braun, 1958, 1962; Steward and Shantz, 1958; Torrey, 1963). That some proliferating cell systems eventually become independent of exogenous IAA and, or, myo-inositol demonstrates the degree of autonomy of which groups of mitotically active cells (i.e. meristems) are capable. This metabolic change appears to accompany a change in organization, e.g. groups of proliferating cells lose their dependence on exogenous sources of IAA at about the same time as a meristem is organized when mitotic divisions become localized and the growth resulting from an increase in cell number and in cell size takes place along one axis.

In all the known systems, however, the presence of a group of mitotically active cells has more functional significance than merely the source of new cells. In roots and in shoots, the meristem controls and determines form: it supplies cells for growth and development, it controls the physiological state and thus potentialities of those cells; and it determines, apparently by controlling the supply of IAA, the pathway of development its progeny cells undergo. On the basis of many results auxin can be described as "an integrative factor in plant growth" (Thimann, 1963) and the way is open for further studies

of interactions between diffusible and non-diffusible growth factors. It is clear, however, that studies of integrating factors in growth are only part of the whole story; there is also an integrating center. That center is the primary apical meristem. The basic functional unit appears to consist of approximately 1000 cells in roots and shoots (Clowes, 1954; Torrey, 1963; Ball, 1948) and its response to experimental treatments has been determined (Clowes, 1954; Davidson, 1961, 1965).

It has been suggested, from several lines of evidence, that meristems produce auxin (Torrey, 1957; Davidson, 1960, 1961). The response of meristematic cells to exogenous IAA, or other growth factors, has not, however, been widely studied. One important parameter of the growth of meristems is the duration of the mitotic cycle of the proliferating cells. It reflects the replication and expression of the total genetic information, nuclear and cytoplasmic, of the cell. We have studied and will report here on the effects of IAA and myo-inositol on cell cycles in root apical meristems.

Material and Methods

1. Myo-inositol. Growing roots of Vicia faba and Allium cepa were treated with aqueous solutions of myo-inositol, 0.0000625 M - 0.25 M, for 3, 6 or 24 hours.

2. IAA. Populations of marked cells were induced in roots of V. faba by treating with colchicine (0.025%) or H^3 -thymidine. The effect of IAA on the mitotic cycle times of these marked populations was determined using 10^{-6} - 10^{-4} M IAA for 3 hours.

All roots were fixed in freshly-mixed and chilled glacial acetic acid - ethyl alcohol, 1/3. Roots were hydrolyzed in 1N HCl, stained with Feulgen's reagent and prepared as squashes. Autoradiographs were prepared using NTB-2 liquid emulsion.

Results and Discussion

Myo-inositol. 1) Chromosome effects. Though the evidence that myo-inositol is involved in cell division is strong (Braun, 1958; Pollard, Shantz and Steward, 1961) no specific role has been defined for this compound. However, it produces certain obvious changes in mitotically active cells. With 0.0000625 M to 0.00625 M solutions, a 3 hour treatment of V. faba and A. cepa produced the following effects:

a. Chromosome contraction: chromosomes are shorter and thicker than normal. This effect is maintained through anaphase.

b. Centric regions may not take their normal form. Thus, they do not appear as typical unstained gaps. In extreme cases, the chromosome apparently lacks a centric region. This effect may

also last through anaphase, though since chromatid movement occurs it is clear that the change at the centromere does not inhibit its function at anaphase.

c) Sister-chromatids adhere laterally. The consequence of this lateral adhesion is to produce an apparently undivided structure; chromosomes that show lateral adhesion of sister-chromatids and the absence of a centric gap look remarkably similar to meiotic structures. Lateral adhesion is, however, temporary and the sister-chromatids separate and undergo anaphase movement.

The adhesion of sister-chromatids has been seen repeatedly. It occurs in cells stained with Feulgen's reagent, which is accepted as a stain specific for DNA, and appears to involve the DNA of the chromosomes. In addition, cells are seen with adhesion between chromosomes; this also involved Feulgen-positive material. Phospholipids contain inositol, stain lightly with Feulgen's reagent under some conditions and have been suggested as chromosome constituents (La Cour, 1955); they may be involved. Whatever the cause, however, myo-inositol is capable of affecting chromosome organization within the duration of one mitotic division. It leads to contraction and the disappearance of the centric constriction and it produces an adhesion by means of a Feulgen-positive material.

Similar chromosomal changes are produced by cyclo-hexanes carrying 2-OH groups (1,2 and 1,4 diols) instead of the 6-OH groups

present in myo-inositol. It appears that all 6-OH groups are not necessary to induce changes in chromosome structure. The diols are also implicated somehow in spindle structure since they can maintain isolated spindles (Kane, 1962). Evidence that myo-inositol may form a complex with nucleoproteins, a possibility suggested by our results, comes from studies on UV induced inactivation of bacteria and virus particles. Inositol was found to reduce their sensitivity to UV, under conditions of low humidity (Webb, 1963).

2) Mitotic Index. Roots of A. cepa were treated with 0.025 M and 0.25 M myo-inositol for 3, 6 and 24 hours. Both treatments resulted in a gradual reduction in mitotic index; with 0.25 M solutions, MI fell from 5.7 to 1.7, and with 0.025 M solutions MI fell from 5.7 to 3.1 after 24 hours. The treatments involve nonphysiological concentrations and the suppression of mitotic activity is not surprising. What is interesting is that root meristems can maintain some mitotic divisions even in the continued presence of 0.25 M myo-inositol.

Myo-inositol occurs in i) various phospholipids and thus in membranes, ii) phytin, the salt of phytic acid and iii) various water soluble complexes (Lardy, 1954). Hypo- or hypervitaminosis results in changes in base composition and cell content of RNA (Posternak, 1966). Thus, myo-inositol is as ubiquitous in distribution as other growth factors and it may have several functions; one function appears to be that it plays some role in the chromosome changes of mitosis.

IAA: Effect on intermitotic time of tetraploid cells

Treatment of roots of V. faba with 0.025% colchicine is followed, sometime later, by the appearance in mitosis of tetraploid cells. Roots were treated with 0.025% colchicine or with colchicine and either 10^{-4} M or 10^{-6} M IAA; all treatments were for 3 hours and roots were fixed for the following 31 hours.

Tetraploid cells appeared in division 15 hours after treatment with colchicine alone and by 18 hours, 15.0% of metaphases were tetraploid (Table 1). After colchicine and 10^{-6} M IAA, there were no tetraploid metaphases at 15 hours and their frequency at 18 hours was only 5.4% (cf. roots treated with colchicine alone). It reached 17% by 21 hours. 10^{-6} M IAA clearly delays the appearance of tetraploid cells in mitosis by approximately 3 hours.

With colchicine and 10^{-4} M IAA tetraploid metaphases first appeared after 18 hours but their frequency remained low ($<2.0\%$) until 25 hours, when it reached 18.8%. This represents a delay of about 7 hours compared with roots treated only with colchicine (Table 1).

Tetraploid cells form by nuclear restitution in colchicine treated cells and this begins about 2 hours after the beginning of a colchicine treatment. The time taken for tetraploid metaphases to appear provides an estimate of the intermitotic time of the first

cells to undergo restitution, complete interphase and re-enter mitosis. The size of the mitotic population affected by colchicine and IAA was approximately identical in all three treatments (Table 2); therefore we would expect that the time of appearance and frequency of tetraploid metaphases should be similar in all 3 sets of roots. IAA treatment affects the tetraploid cells once they form and prolongs the duration of their mitotic cycle. This effect is clearly demonstrated on the first group of tetraploid cells to enter mitosis; 10^{-6} M IAA delays their appearance by 3 hours, 10^{-4} M IAA delays it by about 7 hours. For the tetraploid cells that enter mitosis at later times, other considerations arise and they are discussed elsewhere (Webster and Laviates, 1967).

IAA: Effect on Mitotic Index

The effect of IAA on the duration of the mitotic cycle of the induced tetraploid cells provides evidence, from one population of cells, of what happens generally in the meristem: intermitotic time is extended and the result is a fall in mitotic index. With a 3 hour IAA treatment an effect is evident immediately after treatment, though its intensity varies from experiment to experiment; in every case, however, mitotic index begins to fall in the next few hours. The highest concentration we have used (6.26×10^{-4} M IAA) reduced the mitotic index by 50% 5 hours after a 3 hour treatment (from 6.9 to 3.4) and at 11 and 23 hours the

mitotic index was <0.5 (MacLeod and Davidson, 1966). With weaker concentrations (10^{-5} M), there is the same initial fall in MI but by 9 hours, following a 3 hour treatment, mitotic activity has recovered.

We have estimated that the durations of the phases of the mitotic cycle in roots of V. faba are as follows:

G_1	=	2.5 hours	
S	=	6 hours	
G_2	=	3.5 hours	Total = 14 hours
Mitosis	=	2 hours	

On the basis of these timings it appears that IAA, in the concentrations we have used, has little effect on cells in G_2 during treatment. But mitotic index begins to fall when cells in S or G_1 at the time of treatment would be expected to enter mitosis. To determine the effect of IAA on the passage of cells through G_1 , S and G_2 we have used cells labeled with H^3 -thymidine, since it enables us to mark cells in the S phase of interphase.

IAA: Effect on tritium labeled cells.

Roots were labeled with H^3 -thymidine for 1 hour and then treated with 10^{-5} M IAA for 3 hours. Fixations were made at 3 hour intervals for 27 hours.

Estimates of G_1 , S, G_2 and mitosis can be made using the labeled mitosis curve (Quastler and Sherman, 1958). In meristems labeled

with H^3 -thymidine and then treated with IAA it is apparent that there are increases in the duration of the various phases of the mitotic cycle; the extent of these increases cannot be estimated directly from the labeled mitosis curve, however, because of the IAA induced changes in mitotic index. In order to determine the effects of IAA on the time parameters of the mitotic cycle we have compared absolute frequencies of labeled and unlabeled prophases in treated and control roots, rather than percentage labeled prophases. In this way it is possible to determine which cells are being delayed in their entry into mitosis.

At 3 hours there are 33 prophases per 1000 cells in the IAA treated roots and only 4 prophases are labeled; this compares with 55 prophases, 23 of which are labeled, in control roots. The difference in the number of prophases in IAA-treated roots and controls is almost entirely due to a drop in the number of labeled cells. The unlabeled cells continue to enter prophase. These cells were in G_2 at the time of H^3 -thymidine treatment. At 6 hours, this result is repeated, i.e. there is a fall, in IAA-treated roots, in the number of prophases and again it is due mainly to the failure of labeled cells to reach prophase. The frequency of labeled prophases drops from a control value of 42 per 1000 cells to 18 per 1000 cells, while the frequency of unlabeled prophases is reduced by only 3 per 1000 cells, from 15 to 12.

It is evident within the first 6 hours of a treatment with IAA that although there is a reduction in mitotic index, this is due to a decrease in the number of labeled prophase; there is no reduction in the absolute numbers of unlabeled cells that reach prophase and contribute to the mitotic index. We conclude from these data that a 3 hour treatment with 10^{-5} M IAA does not prevent G_2 cells from completing their interphase and entering prophase. The fall in mitotic index must result from a delay in the passage of cells in S at the time of treatment with H^3 -thymidine to mitosis. It may be that these cells are delayed when they reach G_2 ; this seems unlikely since cells already in G_2 are unaffected. It appears that the labeled populations of cells are held in S or some G_1 cells may be prevented from entering S.

By 9 hours the labeled cells have begun to reach prophase and they constitute the bulk of the cells entering division; the absolute number of prophases is still lower than in controls (Table 3). The numbers of prophases in IAA-treated roots reach control values at 12 hours; however, the relative numbers of labeled and unlabeled stages differ in treated roots and controls. In treated roots there are 59 prophases -- 47 labeled and 12 unlabeled; in controls there are 56 prophases -- 10 labeled and 46 unlabeled. Thus at 12 hours, the number of labeled prophases in treated roots finally reaches the value found in control roots at 6 hours.

The low number of unlabeled prophases at 12 hours in the IAA treated roots (12, compared with 46 in controls) suggests that the IAA had delayed some G_1 cells. These cells would have entered S after treatment with H^3 -thymidine but while the roots were in IAA. These cells would be subject to the same delay as the labeled S cells. It is possible, however, that an IAA treatment may also delay the entry of G_1 cells into S, i.e. it inhibits entry of G_1 cells into S as well as increasing the duration of S.

In considering the effect of IAA (10^{-5} M) on the progress of cells through interphase and into mitosis, two results stand out: 1) the delay does not affect all cells to the same extent, i.e. some labeled cells do reach prophase within 3 hours (Table 3); 2) the initial rate of entry of labeled cells into prophase is about 40% of the control value. This value is maintained at 6 and 9 hours but changes from 9 to 12 hours. By 12 hours the rate of entry of cells into prophase has returned to control values. It appears that IAA delays the cells at the end of S by extending the duration of their S period; therefore, the number of cells passing from S to G_2 is reduced. Cells in earlier parts of S are also delayed but the effect of IAA has worn off before they complete S and by the time these cells pass from S to G_2 , they do so in normal numbers.

We have compared the curves of labeled prophases in control and IAA treated roots and find that the time difference between the

50% intercepts on the descending portions of the two curves is 4.3 hours. This is an estimate of the maximum increase in the duration of S induced by IAA, i.e. the auxin increases S to 10.6 hours, from the control value of 6 hours.

Conclusions

The results reported here show that myo-inositol and IAA induce changes in the actively dividing cells of apical meristems. Both compounds induce their effects within the period of one mitotic cycle or a part of the cycle. Myo-inositol affects mitosis itself and can prolong interphase. IAA extends the duration of S but does not appear to induce any changes in the chromosomes themselves. The changes induced by myo-inositol in chromosome structure do not appear to interfere with the completion of a successful anaphase. They are of interest because they resemble, in several respects, the type of chromosome behavior that occurs naturally in the prophase of meiosis. The mechanism of the changes induced by myo-inositol and cylo-hexane-diols requires further investigation.

Neither myo-inositol nor IAA appears to have a single site of action, a situation which makes it difficult to define their biological roles exactly. In the case of inositol its function as a vitamin is probably related, in part, to its widespread distribution as a structural

unit of membranes (Hawthorne, 1964). IAA and inositol may yet prove to have common sites of action since they can combine in vivo (Nicholls, 1967).

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In three experiments using marked populations of cells we have shown that a 3 hour treatment with IAA (10^{-4} to 10^{-6} M) extends the duration of a mitotic cycle. This result emphasizes, for us, the need to explore the behavior of mitotically active cells in meristems of IAA inhibited roots growing in vitro, in the type of system Street has described (1954) and also to analyze the mechanisms controlling differential rates of division within a meristem. If a 3 hour treatment with 10^{-6} M IAA can extend a mitotic cycle by several hours we would question whether cells with an intermitotic time of 14 hours can be responsible for the synthesis of amounts of IAA that would control mitotic activity in adjacent cells of the meristem, such as quiescent center cells.

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Table 2

hours after treatment	3	9	15	18	21	23	25	27	31
colchicine	0	0	4.3	15.0	21.2	17.2	26.4	41.7	47.5
+ 10^{-6} M IAA	0	0	0	5.4	17.1	20.2	23.0	30.1	53.7
+ 10^{-4} M IAA	0	0	0	<0.5	<0.5	1.7	18.8	19.3	55.0

Percentage frequencies of tetraploid metaphases in roots of V. faba treated for 3 hours with 0.025% colchicine or 0.025% colchicine and 10^{-6} M or 10^{-4} M IAA. 500 metaphases were scored for each fixation.

Table 2

	Colchicine	Colchicine + 10^{-4} M IAA	Colchicine + 10^{-6} M IAA
Mitotic , index	11.9	12.2	13.9
Prophases	41	35	40
Metaphases	78	87	98

Mitotic index and numbers of prophases and metaphases per 1000 cells in roots of V. faba treated with colchicine alone or together with IAA. All solutions contained 0.025% colchicine. Roots were fixed after a 3 hr. treatment.

Table 3

Hours after H ³ -TdR	H ³ -Thymidine		H ³ -Thymidine + IAA	
	No. of prophases	Labelled prophases	No. of prophases	Labelled prophases
3	55 ± 18.5	23 ± 10	33 ± 6.0	4.3 ± 1
6	55 ± 11.8	42 ± 10	30 ± 3.0	18 ± 3
9	55 ± 10.4	24 ± 5	24 ± 1.4	19 ± 6
12	56 ± 12.7	10 ± 6	59 ± 10.6	47 ± 6
15	48 ± 5.3	2 ± 2	-	4*
18	50 ± 2.5	21 ± 2	-	12*
21	60 ± 6.2	33 ± 5	51 ± 9.4	12 ± 4
24	48 ± 7.0	18 ± 3	38 ± 5.8	17 ± 5
27	52 ± 2.0	17 ± 7	59 ± 11.4	19 ± 4

Total number of prophases and number of labelled prophases in roots of V. faba treated with H³-Thymidine (1 µc/ml) for 1 hour. Some roots were then placed in 10⁻⁵ M IAA for 3 hours. Times given are from the end of the H³-Thymidine. Roots at 15 and 18 hours stained poorly. A small number of cells (8 and 24) were actually observed and the values marked * have been calculated on the assumption that the prophase frequency is the same as that of the controls.